

Complete amino acid sequence of the large subunit of the low- Ca^{2+} -requiring form of human Ca^{2+} -activated neutral protease (μCANP) deduced from its cDNA sequence

Kazumasa Aoki⁺, Shinobu Imajoh, Shigeo Ohno, Yasufumi Emori, Morio Koike*, Goro Kosaki* and Koichi Suzuki[†]

*Department of Molecular Biology, The Tokyo Metropolitan Institute of Medical Science and *Tokyo Metropolitan Komagome Hospital, Bunkyo-ku, Tokyo 113, Japan*

Received 19 June 1986

The complete amino acid sequence of the large subunit (catalytic subunit) of human low- Ca^{2+} requiring-calcium-activated neutral protease (μCANP) was deduced from its cDNA base sequence. It is composed of 714 amino acid residues and its sequence is highly homologous to the chicken CANP sequence determined previously. Human μCANP , like chicken CANP, has a clear 4-domain structure, and their fundamental structures are essentially the same, although their Ca^{2+} sensitivities are significantly different. The role of each domain in the Ca^{2+} sensitivity and protease activity of CANP is discussed on the basis of sequence comparison.

Ca²⁺-activated neutral protease Calpain Thiol protease cDNA cloning Sequence homology Ca²⁺

1. INTRODUCTION

Ca^{2+} -activated neutral protease (CANP, calpain, EC 3.4.22.17) is an intracellular thiol protease that is ubiquitously found in tissues of higher animals [1–3]. Two types of CANP exist in mammals, μCANP and mCANP , which respectively require micromolar and millimolar Ca^{2+} for their activity [1–3]. Both CANPs are composed of two subunits, a large (catalytic) (M_r 80 000) one and a small (regulatory) (M_r 28 000) one [1–3]. As the small subunit is common to both CANPs [4,5], the Ca^{2+} requirement of CANP is determined apparently by the large subunit [6]. We have already shown that the large subunit of chicken CANP

consists of four domains, two of which were identified as a thiol protease domain and a calmodulin-like Ca^{2+} -binding domain [7]. Comparison of the structures of μ and mCANPs is important for clarifying the molecular basis of their different Ca^{2+} sensitivities. However, since only one molecular species of CANP, with an intermediate Ca^{2+} sensitivity, has been found in chicken [7–9], we started to analyze the structures of μ and mCANPs from mammalian sources. Recently, cDNA clones corresponding to the C-terminal Ca^{2+} -binding domains of rabbit μ and mCANPs were isolated [10]. Here we report the complete amino acid sequence of the large subunit of human μCANP , which was deduced from the nucleotide sequence of its cDNA clone isolated using rabbit μCANP cDNA as a probe.

2. MATERIALS AND METHODS

Total RNA was prepared from human skeletal

[†] To whom correspondence should be addressed

⁺ Present address: Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162, Japan

muscle and spleen [11], and then poly(A)⁺ RNA enrichment was performed by oligo(dT)-cellulose chromatography [12]. Two cDNA libraries were constructed. One was prepared from human skeletal muscle poly(A)⁺ RNA by the conventional method, as in [7]. The other library was constructed from human spleen poly(A)⁺ RNA. Double-stranded cDNA was synthesized as in [13], and fractionated on low-gelling-temperature agarose gel after *Eco*RI linker ligation. cDNA longer than 2.3 kb was collected and ligated to the vector, λ gt10 [14]. Screening of the library was performed as in [12]. DNA was sequenced according to [15].

3. RESULTS AND DISCUSSION

3.1. Identification and DNA sequence of the cDNA clone for the large subunit of human μ CANP

The cDNA library from human skeletal muscle was screened with a *Pst*I cDNA fragment for the large subunit of rabbit μ CANP [10]. One positive clone (pHM42) was isolated from about 45000 transformants and sequenced. Further screening of about 4×10^5 plaques from the human spleen cDNA λ gt10 library with the pHM42 insert as a hybridization probe yielded 18 positive clones. The clone (λ 31) containing the longest insert was subjected to nucleotide sequence analysis after subcloning into the pUC8 vector (resulting subclone; p31). The restriction map for the cDNA inserts in clones pHM42 and p31 is shown in fig.1,

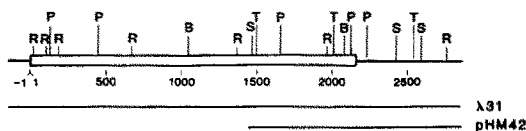


Fig.1. Restriction map of the inserts in cDNA clones p31 and pHM42 for the human μ CANP large subunit. Horizontal lines denote regions covered by the inserts of p31 and pHM42. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the first residue of the ATG triplet that encodes the initiation methionine. An open box indicates the protein-coding region and horizontal lines on both sides of the box indicate the 5'- and 3'-untranslated regions. Vertical lines and letters represent restriction sites. R, *Rsa*I; P, *Pvu*II; B, *Bgl*II; S, *Sma*I; T, *Taq*I.

the total nucleotide sequence of cDNA (3011 bp) being shown in fig.2.

The translational initiation site was assigned to ATG at position 1, because a termination codon, TGA, exists at -57 in phase and the second ATG triplet appears a long way downstream (at position 778). This open reading frame is terminated by TGA at position 2143 and encodes 714 amino acid residues. The calculated M_r (81889) and the amino acid composition agree well with the reported values [16].

The C-terminal amino acid sequence of the large subunit of rabbit μ CANP [10] is almost identical (97% homology) to the deduced sequence for the corresponding position. Furthermore, we have already obtained another cDNA clone that encodes the large subunit of human mCANP (unpublished). Thus, it is concluded that the present cDNA clones encode the large subunit of human μ CANP. Northern blot hybridization of human spleen RNA with the cDNA insert of p31 as a probe gave a single band at a position corresponding to approx. 3.5 kb (fig.3). This indicates that the cloned cDNA, spanning a total of 3011 bp, covers nearly the full length of the μ CANP mRNA.

3.2. Comparison of the amino acid sequences

This is the first complete sequence determination of the large subunit of μ CANP. As for the structure of the large subunit of CANP, the complete sequence of chicken CANP [7] and the C-terminal sequences of rabbit m and μ CANPs [10] have been elucidated.

Comparison of the deduced amino acid sequence of human μ CANP with that of chicken CANP indicated that they are highly homologous, irrespective of the clear difference in their Ca²⁺ sensitivities, and human μ CANP, like chicken CANP, has a clear 4-domain structure (I-IV from the N-terminus). In domain II, the protease domain (amino acid residues 88-327), two active-site amino acid residues, Cys-115 and His-272, are found, and the sequences around these residues are highly conserved among various cysteine proteinases [7,17,18]. In domain IV, the Ca²⁺-binding domain (residues 570-714), 4 consecutive EF-hand sequences are found. The scores for the test sequence [19] are above 11 for all the 4 EF-hand sequences, indicating that these 4 sites can be regarded as potential Ca²⁺-binding sites. The total

-143	AAGGAGAGAGGAGGGCGGAGGG	1201	CTGGATGAGACGGATGACCGGACGACTACCGGGACCGGACTCAGGCTGCAGCTTCGTCG LeuAspGluThrAspAspProAspAspTyrGlyAspArgGluSerGlyCysSerPheVal
-120	CGGAGGGCGGGCGGAGGGCGGGGAGGCGCTCTTCCTGGTTGGCCCTGCCCTGA	1261	CTCGCCCTTATGCAGAAGCACCGCTCGCCGCGAGCGCGCTTCGGCCGCGACATGGAGACT LeuAlaLeuMetGlnLysHisArgArgArgGluArgArgPheGlyArgAspMetGluThr
-60	GCTGCCACCGGGAAGCAGCCTCAGGAGCTGCAGCAGCCCCAACCCCTCCCCCAGG	1321	ATTGGCTTCGGGCTACGAGCTCCCTCGGAGCTGGTGGGCCAGCGCGCGCTACACTTG IleGlyPheAlaValTyrGluValProProGluLeuValGlyGlnProAlaValHisLeu 450
1	ATGTCGGAGGAGATCATCAGCCGGTGTACTGCATGGGGTGCAGCCCAAGTGCAAG MetSerGluGluIleIleThrProValTyrCysThrGlyValSerAlaGlnValGlnLys 1	1381	AAGCGTGACTTCTTCCTGGCCAAATGGCTCGGGCGCGCTCAGAGCAGTTCATCAACCTG LysArgAspPhePheLeuAlaAsnAlaSerArgAlaArgSerGluGlnPheIleAsnLeu
61	CAGCGGGCCAGGAGCTGGCGCTGGGCGGCCATGAGAATGCCATCAAGTACCTGGCCAG GlnArgAlaArgGluLeuGlyLeuGlyArgHisGluAsnAlaIleLysTyrLeuGlyGln	1441	CGAGAGGTGAGCACCCTTCGCCCTGCCACCGGGGATATGTGGTGGTGGCTCCACC ArgGluValSerThrArgPheArgLeuProProGlyGluTyrValIleValProSerThr 500
121	GATTATGAGCAGCTGGGGTGGCATGGCTGCAGAGTGGGACCTCTTCCGTGATGAGGCC AspTyrGluGlnLeuArgValArgCysLeuGlnSerGlyThrLeuPheArgAspGluAla 50	1501	TTGAGGCCCAACAAGGAGGCGGACTTCGTGCTGCGCTTCTCTCAGACAAGAGTGTGGG PheGluProAsnLysGluGlyAspPheValLeuArgPhePheSerGluLysSerAlaGly
181	TTCCCCCGGTACCCAGAGCTGGGTTACAAGGACCTGGGTCCCAATTCTCCAAGACC PheProProValProGlnSerLeuGlyTyrLysAspLeuGlyProAsnSerSerLysThr	1561	ACTGTGGAGCTGGATGACCAGATCCAGGCCAATCTCCCGATGAGCAAGTGTCTCAGAA ThrValGluLeuAspAspGlnIleGlnAlaAsnLeuProAspGluGlnValLeuSerGlu
241	TATGGCATCAAGTGAAGCTCCCGGAACTGCTGCAACCCCCAGTTCATTGTGGAT TyrGlyIleLysTrpLysArgProThrGluLeuLeuSerAsnProGlnPheIleValAsp 100	1621	GAGGACATTGACGAGAATTCAGGCCCTCTTCAGGCAGCTGGCAGGGGAGACATGGAG GluGluIleAspGluAsnPheLysAlaLeuPheArgGlnLeuAlaGlyGluAspMetGlu 550
301	GGAGCTACCCGACAGACATCTGCCAGGGAGCATGGGGGAGTGGCTCTTGGCGGCC GlyAlaThrArgThrAspIleCysGlnGlyAlaLeuGlyAspCysTrpLeuLeuAlaAla	1681	ATCAGCGTGAAGAGTTGCGGCAATCTCAATAGGATCATCAGCAACACAAAGACCTG IleSerValLysGluLeuArgThrIleLeuAsnArgIleIleSerLysHisLysAspLeu 600
361	ATTGCCCTCCCTCACTCTCAACGACACCTCTGCACGAGTGGTTCGCGCGCCAGAGC IleAlaSerLeuThrLeuAsnAspThrGluLeuHisArgValValProHisGlyGlnSer	1741	CGGACCAAGGGCTTCAGCTAGAGTGGCGCGCAGCATGGTGAACCTCATGGATCGTGAT ArgThrLysGlyPheSerLeuGluSerCysArgSerMetValAsnLeuMetAspArgAsp 650
421	TTCCAGAATGGCTATGCCGGCATCTTCCATTTCAGCTGTGGCAATTTGGGAGTGGGTG PheGlnAsnGlyTyrAlaGlyIlePheHisPheGlnLeuTrpGlnPheGlyGluTrpVal 150	1801	GGCAATGGGAAGCTGGGCTGGTGGAGTTCAACATCTCTGGAAACCGCATCCGGAATTAC GlyAsnGlyLysLeuGlyLeuValGluPheAsnIleLeuTrpAsnArgIleArgAsnTyr
481	GACGTGGTCGTGGATGACCTGCTGCCCATCAAGGACGGGAAGCTAGTGTTCGTGCACTCT AspValValValAspAspLeuLeuProIleLysAspGlyLysLeuValPheValHisSer	1861	CTGTCCATCTTCCGGAAGTTTGACCTGGCAAGTGGGCGCAGCATGATGGCTACGAGATC LeuSerIlePheArgLysPheAspLeuAspLysSerGlySerMetSerAlaTyrGluMet
541	GCGGAAGGCAACGAGTCTTGGAGCGCCCTGCTTGAGAAGGCCATGCCAAGTAAATGGC AlaGluGlyAsnGluPheThrPheAlaLeuLeuGluLysAlaTyrAlaLysValAsnGly 200	1921	CGGATGGCCATTGAGTCGGCAGGCTTCAAGCTCAACAAGCTGTACGAGCTCATCATC ArgMetAlaIleGluSerAlaGlyPheLysLeuAsnLysLysLeuTyrGluLeuIleIle 650
601	AGCTACGAGGCGCTGTGAGGGGCGAGCCTCAGAGGCGCTTTGAGGACTTCACAGCGCG SerTyrGluAlaLeuSerGlyGlySerGluGlyPheGluAspPheThrGlyGly	1981	ACCCGCTACTCGGAGCCGACCTGGCGGCTGCACTTTGACAAATTTGCTTGTGCTGGTG ThrArgTyrSerGluProAspLeuAlaValAspPheAspAsnPheValCysCysLeuVal
661	GTTACCGAGTGGTACGAGTTCGCGAAGCTCCAGTGACCTCTACCAGATCATCTCAAG ValThrGluTrpTyrGluLeuArgLysAlaProSerAspLeuTyrGlnIleIleLeuLys	2041	CGGCTAGAGACCATGTTCCGATTTTCAAACTCTGGACACAGATCTGGATGGAGTTGTG ArgGluGluThrMetPheArgPhePheLysThrLeuAspThrAspLeuAspGlyValVal 700
721	GCGCTGGAGCGGGCTCCCTGCTGGCTGCTCCATAGACATCTCCAGCGTCTTAGACATG AlaLeuGluArgGlySerLeuLeuGlyCysSerIleAspIleSerSerValLeuAspMet 250	2101	ACCTTTGACTTGTTAAAGTGGTTCAGCTGACCATGTTTGTGATGAGGACGGGACTCGGT ThrPheAspLeuPheLysTrpLeuGlnLeuThrMetPheAla
781	GAGGCCATCACTTTCAGAAAGTGGTGAAGGGCTATTCCTACTCTGTGACCGGGCCCAAG GluAlaIleThrPheLysLysLeuValLysGlyHisAlaTyrSerValThrGlyAlaLys	2161	CCCCCTGCGTGCTCCCTCCCTCCCTCGTCTGCCAAGCTCGCCTCTACCAACACCACAC
841	CAGGTGAATACCGAGGCGAGGTGGTGAAGCTGATCCGGATGCGGAACCCCTGGGCGAG GlnValAsnTyrArgGlyGlnValSerLeuIleArgMetArgAsnProTrpGlyGlu 300	2221	CAGGCCACCCGACGCAAGTGCCCTTCCTGGAGCAGAGGAGCAGCTCGTCTCTCTGTC
901	GTGGAGTGGACGGGAGCTGGAGGACAGCTCCTCAGAGTGGAAACAAGTGGACCCATAT ValGluTrpThrGlyAlaTrpSerAspSerSerSerGluTrpAsnAsnValAspProTyr	2281	CCCTCTCTCCAGCCACCATCTGCTGCTCGGGCAGAACTGTGTGGCCCTCGCT
961	GAACGGGACAGCTCCGGCTCAAGATGGAGGACGGGAGTTCGTGATCTATTCCGAGAC GluArgAspGlnLeuArgValLysMetGluAspGlyCyluPheTrpMetSerPheArgAsp 350	2341	GTGCCAGCATGGGCTGGGATGGACTCCCTGGGCCCCACCATTCGCAAGCCAGGAAG
1021	TTCATGCGGAGTTCACCGGCTGGAGATCTGCAACCTCACACCGGACGCCCTCAAGAGC PheMetArgGluPheThrArgLeuGluIleCysAsnLeuThrProAspAlaLeuLysSer 350	2401	CAGCTTTCGCTTGTCTGCTCGCTGGGACAGCCCGGGTTTCCCGACATCTGATGTGTG
1081	CGGACCATCCGAAATGGAACACCACTCTACGAAGGACCTGGCGGCGGGGAGCACC ArgThrIleArgLysTrpAsnThrThrLeuTyrGluGlyThrTrpArgArgGlySerThr	2461	CCCTCTCCCACTTTCAGAGGCCACCACTCAGACCAACCGGCTGGCTTGTGCTGAGAC
1141	GCGGGGGCTGCCGAACTACCCAGCCACTTCTGGGTGAACCTCAGTTCAGATCCGG AlaGlyGlyCysArgAsnTyrProAlaThrPheTrpValAsnProGlnPheLysIleArg 400	2521	TATAAATATAACCACTAGCTCGACACAGCTGCGAGTCCAGGCGTGTGGAGCCCTCCC
		2581	GGCTCGGGGAGGCCCCGGGCTGGGAACGCTGTGCTCTCTCGCCGAAGCCACAGCCCC
		2641	CTCTGTCTCTTCTGCGCTGCTGCGGACCAAGAGCTGCCAGCTGTGGGCGCTCGGC
		2701	CTTCCCTCTCTGCTCTTTTATATTATGATTTAAAGGGGACTCTCAGGAGATTG
		2761	TGTACTGGTTATGGGGTGGCAGAGGCACTAGGCTGGGGTGGGAGGTCCCGTGTCCA
		2821	TATAGAGGAACCCCAATATATAAGGCCCCACATCTGTCTGTGA

Fig.2. Nucleotide sequence of the large subunit of human μ CANP cDNA and the deduced amino acid sequence. Two residues at the active site (Cys-115, His-272) are boxed. The amino acid residues corresponding to the Ca^{2+} -binding loop of the 4 EF-hand structures are underlined. Closed triangles indicate the boundaries of domains. The polyadenylation signal is double-underlined.

sequence homology between chicken CANP and human μ CANP is 70%. Furthermore, the homologies of the two proteins are 54, 78, 72 and 65% for domains I, II, III and IV, respectively.

The homology in domain II is higher than those for the other domains.

A clear structural difference was found between the N-terminal sequences of human μ CANP and

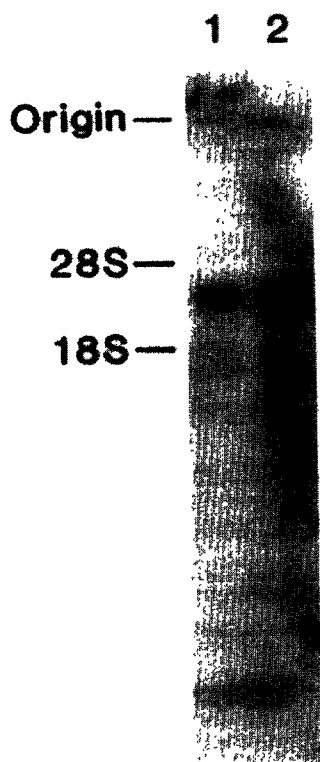


Fig.3. Blot hybridization of human spleen RNA. Poly(A)⁺ RNA (lane 1, ~0.3 μ g) and total RNA (lane 2, ~2.0 μ g) were analyzed by the procedure described in [12] using nylon membrane. The probe used for hybridization was the *Rsa*I fragment (nos 1967–2763 in fig.2) labelled by nick-translation with [α -³²P]dCTP.

The size marker was human rRNA.

chicken CANP. The former is larger than the latter by 7 amino acid residues. The Ca²⁺ sensitivity of chicken CANP increases significantly upon removal of the N-terminal 17-residue peptide by autolysis [7,18]. Thus, the N-terminal region is also important for the Ca²⁺ sensitivity of CANP. In this respect, a clear structural difference in the N-terminal region is noticeable.

From these results we assume that various CANPs have a common fundamental mechanism for the expression of protease activity and that their Ca²⁺ sensitivities are determined mainly by differences in the structure of the C-terminal Ca²⁺-binding domain (domain IV). The N-terminal region (domain I) of native CANP might interact with domains II and/or IV, and thus

repress the Ca²⁺-dependent proteolytic activity. Autocatalytic cleavage of the N-terminal region affects the interaction between functional domains and changes the Ca²⁺ sensitivity of CANP.

ACKNOWLEDGEMENTS

We are grateful to Drs Y. Sukenaga and Y. Shibasaki for providing the λ gt10 cloning system and for useful discussions. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, and Grants-in-Aid for New Drug Development from the Ministry of Health and Welfare, and the National Center for Nervous, Mental and Muscular Disorders of the Ministry of Health and Welfare of Japan.

REFERENCES

- [1] Imahori, K. (1982) in: *Calcium and Cell Function* (Cheung, W.Y. ed.) vol.3, pp.473–485, Academic Press, New York.
- [2] Murachi, T. (1983) in: *Calcium and Cell Function* (Cheung, W.Y. ed.) vol.4, pp.377–410, Academic Press, New York.
- [3] Suzuki, K., Kawashima, S. and Imahori, K. (1984) in: *Calcium Regulation in Biological Systems* (Ebashi, S. et al. eds) pp.213–226, Academic Press, New York.
- [4] Yumoto, N., Kikuchi, T., Sasaki, T. and Murachi, T. (1984) *J. Biochem.* 96, 1531–1537.
- [5] Kawasaki, H., Imajoh, S., Kawashima, S., Hayashi, H. and Suzuki, K. (1986) *J. Biochem.* 99, 1525–1532.
- [6] Kikuchi, T., Yumoto, N., Sasaki, T. and Murachi, T. (1984) *Arch. Biochem. Biophys.* 243, 639–645.
- [7] Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984) *Nature* 312, 566–570.
- [8] Kawashima, S., Nomoto, M., Hayashi, M., Inomata, M., Nakamura, M. and Imahori, K. (1984) *J. Biochem.* 95, 95–101.
- [9] Suzuki, K., Ohno, S., Imajoh, S., Emori, Y. and Kawasaki, H. (1985) *Biomed. Res.* 6, 323–327.
- [10] Emori, Y., Kawasaki, H., Sugihara, H., Imajoh, S., Kawashima, S. and Suzuki, K. (1986) *J. Biol. Chem.* 261, 9465–9471.
- [11] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) *Biochemistry* 18, 5294–5299.
- [12] Maniatis, T., Fritsch, E.F. and Sambrook, J.

- (1982) in: *Molecular Cloning*, Cold Spring Harbor Laboratory, NY.
- [13] Gubler, U. and Hoffman, B.J. (1983) *Gene* 25, 263–269.
- [14] Huynh, T., Young, R. and Davis, R.W. (1985) in: *DNA Cloning, A Practical Approach* (Glover, D. ed.) vol.1, pp.49–78, IRL Press, London.
- [15] Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.* 65, 499–560.
- [16] Hatanaka, M., Kikuchi, T. and Murachi, T. (1983) *Biomed. Res.* 4, 381–388.
- [17] Suzuki, K., Hayashi, H., Hayashi, T. and Iwai, K. (1983) *FEBS Lett.* 152, 67–70.
- [18] Suzuki, K., Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H. and Kisaragi, M. (1985) in: *The Biological Role of Proteinases and Their Inhibitors in Skin* (Ogawa, H. et al. eds) pp.111–120, University of Tokyo Press, Tokyo.
- [19] Tufty, R.M. and Kretsinger, R.H. (1975) *Science* 187, 167–169.